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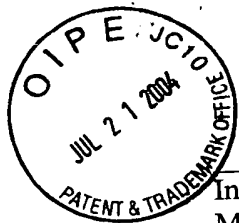
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Martin M. Matzuk et al.

Application No.: 09/830,810

Art Unit: 1653

Filed: October 28, 1999

Examiner: Desai, Anandu U

For: OVARY-SPECIFIC GENES AND PROTEINS

1.132 DECLARATION OF DR. MARTIN M. MATZUK

1. I, Martin M. Matzuk, do hereby declare and state the following:
2. I am a professor in the Department of Pathology at Baylor College of Medicine. I am skilled in the fields of fertility and embryonic development. (See attached Curriculum Vitae).
3. I am one of the inventors, and I have read the above-captioned patent application, as well as all Examiner's Actions and responses.
4. In the present application, myself and the Pei Wang isolated a polynucleotide sequence, SEQ.ID.NO.1. that was specifically and only expressed in the oocytes. This sequence is known as O1-180 and zygote arrest 1 (*Zar1*). Since the expression of this protein was similar to GDF-9, we assumed that it would have a similar function.
5. We performed several experiments to determine the function of *Zar1*. Such experiments included producing transgenic mice lacking *Zar1* or knockout mice (*Zar1*^{-/-}). The *Zar1*^{-/-} females were infertile compare to *Zar1*^{+/+} and *Zar1*^{+/-} females. (See page 188, second column of Wu et al., Nature Genetics 33:187-191, 2003).
6. I assert that the asserted utility in the patent application is a credible utility based upon our recent evidence as indicated in Wu et al., Nature Genetics 33:187-191, 2003.

7. I hereby declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Title 18 § 1001 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

7-19-04

Date

Martin M. Matzuk

Martin M. Matzuk, M.D., Ph.D.

CURRICULUM VITAE

NAME: Martin Matthew Matzuk, M.D., Ph.D.
ADDRESS: Department of Pathology
 One Baylor Plaza
 Houston, TX 77030

TELEPHONE: (713) 798-6451
FAX: (713) 798-5833
E-MAIL: mmatzuk@bcm.tmc.edu

DATE OF BIRTH: January 30, 1960
PLACE OF BIRTH: New Brunswick, New Jersey

EDUCATION: University of Chicago, B.A. with Honors, 1982 (Biology)

Washington University, M.D., Ph.D., 1989
 Thesis: Structure-function studies of the glycoprotein hormones using mutagenesis,
 chimeric genes, and gene-transfer
 (Advisor, Dr. Irving Boime; Department of Pharmacology)

PROFESSIONAL EXPERIENCE:

Undergraduate Research Assistant, University of Chicago, Dept. of Biochemistry
 (Advisor, Dr. Nicholas R. Cozzarelli), 1980-1982

Staff Research Associate, University of California, Berkeley, Dept. of Molecular Biology
 (Advisor, Dr. Nicholas R. Cozzarelli), 1982-1983

Teaching Assistant, Washington University School of Medicine,
 Pharmacology, 1985-1986

Clinical Pathology Resident, Dept. of Pathology and Laboratory Medicine,
 Hospital of the University of Pennsylvania, 1990-1991

Pathology Resident and Postdoctoral Fellow, Dept. of Path. and Molecular Genetics,
 Baylor College of Medicine, 1991-1993

Clinical Instructor, Dept. of Pathology, Baylor College of Medicine, 1992-1993

Director of Clin. Chem. and Diagnostic Immunol., Ben Taub Gen. Hosp., 1993-present

Assistant Professor, Departments of Pathology, Molecular and Human Genetics,
 and Molecular and Cellular Biology, Baylor College of Medicine, 1993-1995

Associate Professor, Departments of Pathology, Molecular and Human Genetics,
 and Molecular and Cellular Biology, Baylor College of Medicine, 1995-1998

Professor, Departments of Pathology, Molecular and Human Genetics,
 and Molecular and Cellular Biology, Baylor College of Medicine, 1998-present

Stuart A. Wallace Chair in Pathology,
 Baylor College of Medicine, 1999-present

HONORS:

Edmondson Research Fellowship, University of Chicago, 1981
Medical Scientist Training Program, Washington University, 1983-1989
Spencer T. and Ann W. Olin Fellowship, Washington University, 1989
The Dr. Philip Needleman Prize For Excellence in Pharmacology Research;
Washington University, 1990
Resident Award at the 5th Annual Scientific Symposium of Alumni and
Friends, Baylor College of Medicine, 1992
John R. Rainey, MD, Resident Award at the 72nd Annual Meeting of the
Texas Society of Pathologists, 1993
Experimental Pathologist-in-Training Award, American Society for Investigative
Pathology, 1993
The Michael E. DeBakey, M.D., Excellence in Research Award,
Baylor College of Medicine, 1995
The Richard E. Weitzman Memorial Award, The Endocrine Society, 1996
Inaugural Ernst Knobil Lecture, University of Pittsburgh, 1998
The Bruce Stewart Memorial Lecture, American Society for
Reproductive Medicine, 1998
The HypoCCS Award, Eli Lilly, 1999
O'Neal/Spjut Award, Baylor College of Medicine, 2001
NIH MERIT Award, RO1HD33438, May 1, 2001
Pfizer Outstanding Investigator Award, American Society for Investigative
Pathology, 2002
The Society for the Study of Reproduction Research Award, 2002

TEACHING AND ADMINISTRATIVE RESPONSIBILITIES:

Co-director, Medical Scientist Training Program, 1995-present
Member, Medical Scientist Training Program Operating Committee, 1993-present
Faculty Member, Developmental Biology Program, 1994-present
Member, Animal Research Committee, 1994-present
Instructor, Mammalian Molecular Genetics, 1993-1996
Instructor, Developmental Biology I, 1994-1996
Instructor, Reproductive Biology, 1997-present
Laboratory Instructor, Medical School Pathology, 1994-1995
Instructor, Pathology Resident Didactic Lecture Series, 1998-present
Advisor, Graduate and Medical Students (Nine), 1994-present
Member, Graduate Student Thesis Committees (Thirty-six), 1993-present
Member of the Medical Staff, Ben Taub General Hospital, 1993-present
Member of the Medical Staff, The Methodist Hospital, 1994-present
Member, BaylorMedCare, 1997-1999
Member, Faculty Search Committee, Molecular and Cellular Biology, 1995-1996
Member, Faculty Search Committee, Molecular & Human Genetics, 1996-1997
Member, Faculty Search Committee, Pathology, 1996-1997, 1997-1998
Member, Intradepartmental Appointments and Promotions Committee, 1999-present
Member, Standing Examination Committee, Molecular and Cellular Biology, 2000-present
present
Member, Standing Examination Committee, Molecular and Human Genetics, 2002-present

EXTERNAL THESIS ADVISORY COMMITTEES:

“Thesis defense opponent”
Dr. Kirsi Kanaanan
Student of Dr. Ilpo Huhtaniemi
Turku University, Finland, 1996

“Thesis examiner”
Dr. Shyr-Yeu Lin
Student of Dr. David DeKretser
Monash University, Australia, 2003

AD HOC REVIEWER:

Funding Agencies:

NIH, National Institute of Child Health and Human Development
NIH, National Cancer Institute
The Lalor Foundation
March of Dimes
Marsden Fund, The Royal Society of New Zealand
Michigan Diabetes Research and Training Center, Pilot/Feasibility Grants
Michael Smith Foundation for Health Research
MINERVA Cooperative Germany/Israel Research Grants
National Health and Medical Research Council, Australia
National Science Foundation
The New Zealand Neurological Foundation
North Carolina Biotechnology Center
Research Grants Council of Hong Kong
United States Department of Defense
United States Department of Agriculture
The Wellcome Trust

AD HOC REVIEWER:

Journals:

American Journal of Pathology
Archives of Medical Research
Biochimica et Biophysica Acta
Biotechniques
Biology of Reproduction
Cell
Cytokine
Development
Developmental Biology
Developmental Dynamics
The EMBO Journal
Endocrine
Endocrine Reviews
Endocrinology
European Journal of Endocrinology
Gene
Genes and Development
Genome Research
Genomics
Human Reproduction
Human Reproduction Update
Journal of Andrology
Journal of Cell Science
Journal of Clinical Endocrinology and Metabolism
Journal of Clinical Investigation
Journal of Endocrinology
Journal of Hepatology
Mammalian Genome
Mechanisms of Development
Molecular and Cellular Biology
Molecular and Cellular Endocrinology
Molecular Endocrinology
Molecular Human Reproduction
Molecular Reproduction and Development
Nature
Nature Genetics
Nature Medicine
Nature Neuroscience
Nature Reviews Molecular Cell Biology
New England Journal of Medicine
Oncogene
Proceedings of the National Academy of Sciences
Proceedings of the Society for Experimental Biology and Medicine
Reproduction
Science
Trends in Endocrinology and Metabolism
Trends in Genetics

INVITED LECTURES:

UCLA Symposia on Molecular and Cellular Biology, Molecular Biology of Intracellular Protein Sorting and Organelle Assembly, Workshop, Taos, New Mexico, January 30-February 5, 1987

Gordon Research Conference, Reproductive Tract Biology, Plymouth, New Hampshire, July 6-10, 1992

European Science Foundation Scientific Program on Developmental Biology, Workshop on the Transforming Growth Factor β Family, Leuven, Belgium, November 19-21, 1992

Fifteenth Annual Workshop on Cell and Molecular Techniques, "Techniques in Cellular Signaling and Hormone Action", Houston, Texas, February 28-March 4, 1993

Southeast Regional Developmental Biology Conference, Athens, Georgia, May 14-16, 1993

Seventy-Fifth Meeting of the Endocrine Society, Plenary Session, "Gene Deletions and Endocrine Diseases", Las Vegas, Nevada, June 9-12, 1993

Eighth European Workshop on Molecular and Cellular Endocrinology of the Testis, De Panne, Belgium, March 27-31, 1994

Fifth International Workshop on Multiple Endocrine Neoplasia, Stockholm, Sweden, June 29-July 2, 1994

Serono Symposia, Tenth Ovarian Workshop: Frontiers in Ovarian Research, Ann Arbor, Michigan, July 21-23, 1994

International Workshop on Adrenal Glands, Vascular System, and Hypertension, Hong Kong, April 20-22, 1995

Third Conference of the International Union of Biochemistry and Molecular Biology, Singapore, April 23-27, 1995

24th Annual Reproductive Science Centers Director's Meeting, "Reproductive Developmental Biology", sponsored by the Reproductive Sciences Branch of the Center for Population Research, NICHD, San Francisco, July 14-15, 1995

Recent Progress in Hormone Research, 51st Conference, Stevenson, Washington, July 29-August 3, 1995

Serono Symposia, Life Cycle of the Ovarian Follicle, Fort Lauderdale, Florida, November 2-5, 1995

INVITED LECTURES (cont):

Keystone Symposia on Molecular & Cellular Biology,
"Molecular and Developmental Biology of the Extracellular Matrix",
Keystone Colorado, January 5-11, 1996

Triangle Consortium for Reproductive Biology, "The Development of the
Reproductive Systems", Keynote Address,
Chapel Hill, North Carolina, January 20, 1996

The American Association for the Advancement of Science
Annual Meeting and Science Innovation Exposition,
Baltimore, Maryland, February 8-13, 1996

The 25th Anniversary of the National Institutes of Child Health
and Human Development Centers' Program,
NICHD Molecular Endocrinology Workshop,
Houston, Texas, May 21, 1996

Gordon Research Conference, Reproductive Tract Biology,
Plymouth, New Hampshire, July 7-12, 1996

29th Annual Meeting of the Society for the Study of Reproduction,
London, Ontario, July 27-30, 1996

Reproductive Endocrine Symposium, "Ovarian Messengers",
University of Louisville, Louisville, Kentucky, September 27, 1996

3rd International Symposium on Biomedical Diagnostic and Prognostic Indicators,
International Society for Preventative Oncology,
Nice, France, October 26-28, 1996

Ares-Serono International Symposium on "Inhibin, Activin, and Follistatin...
Recent Advances and Future Views",
Tokushima, Japan, Nov. 9-10, 1996

HHMI-Sponsored Workshop on "Germ line Development"
Chevy Chase, Maryland, December 1-4, 1996

American Society of Andrology, Annual Meeting,
Baltimore, Maryland, February 23-25, 1997

"Hormones and Men's Health", Monash Medical Center and Prince Henry's Institute,
Keynote Speaker, Melbourne, Australia, March 19-21, 1997

Ovarian Cancer Biology Workshop, National Cancer Institute,
Bethesda, Maryland, April 6-8, 1997

Vth International Congress of Andrology,
Salzburg, Austria, May 25-29, 1997

INVITED LECTURES (cont):

Seventy-Ninth Meeting of the Endocrine Society, Plenary Session,
"Genetic Determination of Gonadal Development",
Minneapolis, Minnesota, June 11-14, 1997

Bohan Visiting Scientist Lecture,
University of Kansas Medical Center
Kansas City, Kansas, July 14-15, 1997

American Urological Association Summer Research Conference,
"Cell Biology of the Testis: Cell-Cell Interactions",
Houston, Texas, August 8-10, 1997

Comitato Promotore Telethon,
Telethon Comatti La Distrofia Muscolare E LE Altre Malattie Genetiche,
Bologna, Italy, November 16-18, 1997

"Defining Cytokine Biology through Knockout and Transgenic Mouse Models",
The National Cancer Institute,
December 11, 1997

Society for Gynecological Investigation, Annual Meeting Course,
"How to find a gene and beyond...",
Atlanta, Georgia, March 11-14, 1998

"Reproduction in the 21st Century", International Symposium
Sponsored by the Universidad de la Salle,
Bogota, Columbia, March 13, 1998

Sixth Congress of the International Society of Gynecological Endocrinology,
Plenary Session, "Molecular Biology in Gynecological Endocrinology",
Crans-Montana, Switzerland, March 18-22, 1998

"First International Conference on the Genetic Origins of Premature Ovarian Failure",
Sponsored by the Section on Women's Health,
Developmental Endocrinology Branch,
National Institute of Child Health and Human Development,
Washington, D.C., April 2-4, 1998

Genetics Institute Discovery Research Retreat, "Signal Transduction",
Ogunquit, Maine, May 20-22, 1998

"Frontiers in Reproduction: Molecular and Cellular Concepts and Applications",
Woods Hole, Massachusetts, June 24-27, 1998

5th International Pituitary Congress,
Naples, Florida, June 28-30, 1998

INVITED LECTURES (cont):

Society for the Study of Fertility, Annual Meeting,

Plenary Session, "Gonadal Development and Function"
Glasgow, Scotland, July 6-8, 1998

XIIth Ovarian Workshop, Plenary Session,
"Follicular Development and Oocyte Maturation"
Houston, Texas, August 5-7, 1998

31st Annual Meeting of the Society for the Study of Reproduction,
Techniques Workshop,
"Gene Knockouts: Practical Considerations and Expectations",
College Station, Texas, August 8, 1998

"The Biology of Menopause",
Sponsored by the National Institute on Aging and Serono,
Newport Beach, California, September 10-13, 1998

Inaugural Ernst Nobil Lecture, The University of Pittsburgh,
Center for Research in Reproductive Physiology,
Pittsburgh, Pennsylvania, September 16-17, 1998

37th Annual Meeting of the European Society for Paediatric Endocrinology,
Plenary Lecture, Florence, Italy, September 24-27, 1998

Cold Spring Harbor Meeting, Germline Development,
Cold Spring Harbor, New York, October 1-3, 1998

16th World Congress on Fertility and Sterility and American Society for Reproductive
Medicine, 54th Annual Meeting, "Bruce Stewart Memorial Lecture",
San Francisco, California, October 4-8, 1998

4th International Dahlem Symposium, "Cellular Signal Recognition and Transduction",
Freie Universitat Berlin, Berlin, Germany, October 8-10, 1998

Annual Meeting of the American Society for Gravitational and Space Biology,
Plenary Lecture, Houston, Texas, October 28-31, 1998

Workshop on the Regulation of Reproductive Function,
Annual Meeting of the Israel Fertility Association,
Jerusalem, Israel, April 12-14, 1999

The Third Annual HypoCCS Symposium, "Regulation of Pituitary Hormone Secretion"
Venice, Italy, April 15-17, 1999

1999 North American Inhibin and Activin Congress,
Chicago, Illinois, May 21-23, 1999

INVITED LECTURES (cont):

International Workshop on Early Folliculogenesis and Oocyte Formation, Development and Arrest: Basic and Clinical Aspects, Ares-Serono Foundation,
London, England, June 10-11, 1999

32nd Annual Meeting of the Society for the Study of Reproduction,
Pullman, Washington, July 31-August 3, 1999

Genetic Regulation of Gametogenesis and Development, The Royal Society of
Edinburgh,
Edinburgh, Scotland, September 23-24, 1999

“Reproductive Sciences 2000: Technology in the Service of Biology”,
The Annual Meeting of the Society of Gynecologic Investigation,
Salt Lake City, Utah, February 23-26, 2000

“The Ovary: from Organogenesis to Function to Failure”
Sponsored by the Section on Women’s Health
Developmental Endocrinology Branch
National Institute of Child Health and Human Development,
Washington, D.C., March 30-31, 2000

“Molecular Laboratory Research Course for Clinical Endocrinologists”,
The University of Hong Kong
Hong Kong, May 18-20, 2000

“Molecular Medicine for the Practicing Clinician”,
Centre of Endocrinology and Diabetes
Hong Kong, May 21, 2000

“Embryogenesis Begins During Oogenesis. Follicle Development and Oocyte Fate”,
Bologna, Italy, May 26-27, 2000

Frontiers in Reproduction Symposium, Oocytes and Human Reproduction,
“Egg Futures: 2000 and Beyond”
Boston, Massachusetts, June 15-17, 2000

Eighty-Second Meeting of the Endocrine Society, Plenary Session,
“Regulation of Inhibin/Activin Action in Reproduction”,
Toronto, Canada, June 21-24, 2000

Gordon Research Conference, Gametogenesis and Embryogenesis,
New London, Connecticut, July 2-6, 2000

Ares-Serono Foundation Workshop, “Molecular Genetics of Human Reproduction”
Heronissos Beach, Crete, September 15-17, 2000

Round Table Conference, “Alternative Approaches to IVF”,
Sponsored by N.V. Organon,
Lisbon, Portugal, September 22-23, 2000

INVITED LECTURES (cont):

“Inhibins, Activins, and Follistatin”, Ares-Serono Symposia,
Melbourne, Australia, October 26-28, 2000

11th Congress of the International Society for Endocrinology,
Sydney, Australia, October 29-November 3, 2000

“Montreal Research Day”,
Sponsored by the University of Montreal and McGill University,
Montreal, Canada, January 17, 2001

European Society of Human Reproduction and Embryology
“Mammalian oogenesis and folliculogenesis: in vivo and in vitro approaches”,
Lisbon, Portugal, April 6-7, 2001

XIIth International Workshop on the Development and Function of
the Reproductive Organs, Ares-Sorono Foundation,
Jerusalem, Israel, April 30-May 3, 2001

“First Annual Advances in Contraceptive Help”,
Sponsored by Wyeth Ayerst Pharmaceuticals
Charleston, South Carolina, May 20-22, 2001

17th Annual Meeting of the European Society of Human Reproduction and Embryology,
Lausanne, Switzerland, July 1-4, 2001

FASEB Conference, “The TGF- β superfamily: signaling and development”,
Tucson, Arizona, July 7-12, 2001

7th Annual Mouse Developmental Genetics Course,
Albert Einstein College of Medicine
Bronx, NY, August 26 – September 1, 2001

Symposium for the Inauguration of The Weitzmann Women’s Health Research Center,
“Fertility and Gender-Specific Cancer”,
Rehovot, Israel, November 11, 2001

Ernst Schering Research Foundation Workshop
“The Future of the Oocyte: Basic and Clinical Aspects”
Berlin, Germany, January 30 - February 1, 2002

21st Joint Meeting of the British Endocrine Societies,
Harrogate, United Kingdom, April 8-11, 2002

Colloque 2002 de la Société Française de Génétique,
“Reproduction et lignée germinale: génétique et pathologies, éthique”
Pasteur Institute, Paris, France, April 10-11, 2002

INVITED LECTURES (cont):

Experimental Biology 2002,
"Translating the genome",
American Society for Investigative Pathology
New Orleans, Louisiana, April 20-24, 2002

Texas Forum of Female Reproduction,
Keynote Speaker,
Houston, Texas, May 2-3, 2002

CEJKOVICE 2002, "From Oocyte to Embryonic Stem Cell:
A Lesson from Pluripotency",
Southern Moravia, Czech Republic, June 6 - 9, 2002

Gordon Research Conference, Gametogenesis and Embryogenesis,
New London, Connecticut, June 30-July 4, 2002

35th Annual Meeting of the Society for the Study of Reproduction,
Baltimore, Maryland, July 28-31, 2002

5th Copenhagen Workshop on Carcinoma in situ and Cancer of the Testis,
Copenhagen, Denmark, August 29-31, 2002

TuBS Symposium on Genetic Engineering of Mice for Biology and Disease Models
Mauno Koivisto Center, BioCity
Turku, Finland, November 28-29, 2002

Testis Workshop, "Functional Genomics of Male Reproduction"
Phoenix, Arizona, March 26-29, 2003

Inaugural speaker, "Billie A. Field Memorial Lectureship",
College of Veterinary Medicine,
University of Illinois at Urbana – Champaign
Urbana, Illinois, April 22, 2003

The Twenty-Second Annual University of Kentucky Symposium in
Reproductive Sciences, "Functional Genomics and Mammalian Reproduction"
Lexington, Kentucky, May 16-17, 2003

International Conference on the Female Reproductive Tract,
Monastery of Seeon, Germany, May 30-June 2, 2003

Ares-Serono Workshop on Human Implantation, "Genomics/Proteomics of the ovary",
Madrid, Spain, June 28, 2003

"State of the A.R.T." meeting titled "Communication between oocyte and ovary",
Madrid, Spain, June 28-29, 2003

INVITED LECTURES (cont):

International Ares-Serono Workshop on "Inhibins, Activins, and Follistatins"
Siena, Italy, July 3-4, 2003

FASEB Conference, "TGF- β Superfamily: Signaling and Development",
Tucson, Arizona, July 12-17, 2003

Eppigenetic contribution to our understanding of the oocyte,
The Jackson Laboratory
Bar Harbor, Maine, August 2-3, 2003

59th Annual Meeting of the American Society for Reproductive Medicine,
"Reproductive Medicine and ART: Basic Science and Future Promise"
San Antonio, Texas, October 11-15, 2003

Cytokine-regulated gene expression at the crossroads of innate immunity,
inflammation and fertility
New York University School of Medicine
New York City, October 17-18, 2003

Annual meeting, Taiwanese Society for Reproductive Medicine
Taipei, Taiwan, December 20-21, 2003

Reproductive Medicine Conference "Updates in Infertility Treatment"
Marco Island, Florida, January 22-24, 2004

Annual Meeting of the "Hinterzartener Kreis fuer Krebsforschung/Cancer Research"
"Growth factors, Repair and Cancer"
Cadenabbia, Italy, April 22-25, 2004

Gordon Research Conference, Gametogenesis and Embryogenesis,
New London, Connecticut, June 6-11, 2004

XIII International Workshop on the Development and Function of the
Reproductive Organs
Copenhagen, Denmark, June 12-16, 2004

37th Annual Meeting of the Society for the Study of Reproduction,
Minisymposium on "Oocyte-somatic cell interactions in folliculogenesis",
Vancouver, British Columbia, August 1-4, 2004

Third International Symposium "Assisted Conception and Reproductive Biology:
Two Perspectives, One Vision"
Sponsored by Technobios Procreazione
"Maternal effect genes during preimplantation development",
Bologna, Italy, November 18-20, 2004

GRANTS AWARDED (* = Current Support):

National Institutes of Health, National Institute of Child Health and Human Development; K11HD00960;
"Developmental and Functional Roles of Activin and Inhibin";
August 1, 1991-July 31, 1996. I declined years 4-5.

National Institutes of Health, National Institute of Child Health and Human Development; NRSA F32HD07539;
"Developmental and Functional Roles of Activin and Inhibin";
Awarded May 1991; I declined.

The Lalor Foundation; "Developmental and Functional Roles of Activin and Inhibin in Mammalian Reproduction";
June 1, 1991-May 31, 1992; Renewal June 1, 1992-May 31, 1993

*National Institutes of Health, National Institute of Child Health and Human Development; R01HD32067;
"Functional Analysis of Activins During Development";
August 1, 1994-July 31, 1997; Renewal December 1, 1998 – May 31, 2004;
Renewal June 1, 2004-March 31, 2009

*National Institutes of Health, National Cancer Institute; R01CA60651;
"Mouse Models to Study Gonadal Tumor Development";
August 1, 1993-May 31, 1997; Renewal June 1, 1997 - March 31, 2001;
Renewal April 1, 2001 – March 31, 2006

*National Institutes of Health, National Institute of Child Health and Human Development; R37 HD33438;
"Analysis of Reproductive Function Using Transgenic Mice";
May 1, 1996-April 30, 2001; Renewal May 1, 2001 – April 30, 2006

*National Institutes of Health, National Institute of Child Health and Human Development; U54 HD07495 (PI, Dr. Bert O'Malley);
"Center for Reproductive Biology Research Grant";
April 1, 1996-March 31, 1998; Renewal April 1, 1998 - March 31, 2004;
Renewal April 1, 2004 – March 31, 2009 (years 32-36)

Genetics Institute, Cambridge Massachusetts; "Functional Analysis of Transforming Growth Factor β Superfamily Members - I";
October 1, 1996-March 31, 1999; Renewal July 1, 1999 – June 30, 2000

Metamorphix, Baltimore, Maryland; "Functional Analysis of Transforming Growth Factor β Superfamily Members - II";
October 1, 1996-September 30, 1997;
Renewal February 1, 1998 - January 31, 1999

National Institutes of Health, National Institute of Child Health and Human Development; RO3 HD37231;
"Identification and Analysis of Novel Ovarian Genes";
December 15, 1998 - November 30, 2000

GRANTS AWARDED, continued:

*Wyeth-Ayerst, Women's Health Research Institute;
"Analysis of Putative Female Infertility-Associated Genes";
April 1, 1999 – March 31, 2003; Renewal April 1, 2003 – March 31, 2004

Moran Foundation, Lia Lechago Fund for Cardiovascular Research
July 1, 1999 – June 30, 2000

*National Institutes of Health, National Institute of Child Health and Human
Development; R01 HD42500;
"Regulation of Key Processes in Oocyte Biology"
April 1, 2003 – March 31, 2008

ADVISORY/EDITORIAL BOARDS, CHAIR, AND CONSULTANT ACTIVITIES:

Children's Nutrition Research Center Advisory Board, Baylor College of Medicine,
Houston, Texas, 1997-present

Research Consultant, Genetics Institute, Cambridge, Massachusetts, 1996-2000

Advisory Committee, The Oregon Regional Primate Research Center,
The Oregon Health Sciences University, Portland, Oregon, 1998-present

Research Consultant, Wyeth-Ayerst Research, 1999-present

Advisory Committee, Career Awards in the Biomedical Sciences,
Burroughs Wellcome Fund, 1999-present

Editorial Board, Journal of Endocrinology, 2001-present

Editorial Board, Molecular Endocrinology, 2001-present

Committee on New Frontiers in Contraceptive Research,
Institute of Medicine/National Academy of Sciences,
Board on Health Science Policy, 2003-2004

Co-Chair, Advisory Committee, Career Awards in the Biomedical Sciences,
Burroughs Wellcome Fund, 2004-2006

Co-Chair, 3rd Advances in Contraceptive Health Conference,
Sponsored by Wyeth Research, Tucson, AZ
February 22-24, 2004

ELECTED SOCIETIES:

Sigma Xi, 1982
The Endocrine Society, 1991-present
American Society for Investigative Pathology, 1993-present
Society for the Study of Reproduction, 1999-present

PATENTS:

Boime, I. and Matzuk, M.M. "Modified Forms of Reproductive Hormones";
U.S. Patent and Trademark Office; Patent Number 5,177,193; Issued January 5, 1993.

Boime, I. and Matzuk, M.M. "DNA encoding reproductive hormones and expression
using a minigene"; U.S. Patent and Trademark Office; Patent Number 5,405,945;
Issued April 11, 1995.

Boime, I., Matzuk, M.M., and Keene, J.L. "Follicle stimulating hormone-glycosylation
analogs", U.S. Patent and Trademark Office, Patent Number 6,306,654;
Issued October 23, 2001

Wakil, S., Matzuk, M.M., and Abu-Elheiga, L. "ACC2-knockout mice and uses thereof",
U.S. Patent and Trademark Office; Patent Number 6,548,738;
Issued April 15, 2003

Matzuk, M.M., Elvin, J.A., and Wang, P. "Assay for growth differentiation factor 9",
U.S. Patent and Trademark Office, Patent Number 6,680,174;
Issued January 20, 2004

Wakil, S.J., Matzuk, M.M., and Abu-Elheiga, L. "Acetyl-coenzyme a carboxylase 2 as a target in
the regulation of fat burning, fat accumulation, energy homeostasis, and insulin action",
U.S. Patent and Trademark Office; Patent Number 6,734,337;
Issued May 11, 2004

PUBLICATIONS:

1. Dean, F., Krasnow, M.A., Otter, R., **Matzuk, M.M.**, Spengler, S.J., and Cozzarelli, N.R. *Escherichia coli* type-1 topoisomerases: identification, mechanism, and role in recombination. Cold Spring Harbor Symposia on Quantitative Biology, Vol. 47, 769-777 (1983).
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Zygote arrest 1 (*Zar1*) is a novel maternal-effect gene critical for the oocyte-to-embryo transition

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The female gamete (the oocyte) serves the distinct purpose of transmitting the maternal genome and other maternal factors that are critical for post-ovulation events^{1–4}. Through the identification and characterization of oocyte-specific factors, we are beginning to appreciate the diverse functions of oocytes in ovarian folliculogenesis, fertilization and embryogenesis^{5,6}. To understand these processes further, we identified genes called zygote arrest 1 (*Zar1* and *ZAR1* in mouse and human, respectively) as novel oocyte-specific genes. These encode proteins of 361 amino acids and 424 amino acids, respectively, which share 59% amino-acid identity and an atypical plant homeo-domain (PHD) motif⁷. Although *Zar1*-null (*Zar1*^{−/−}) mice are viable and grossly normal, *Zar1*^{−/−} females are infertile. Ovarian development and oogenesis through the early stages of fertilization are evidently unimpaired, but most embryos from *Zar1*^{−/−} females arrest at the one-cell stage. Distinct pronuclei form and DNA replication initiates, but the maternal and paternal genomes remain separate in arrested zygotes. Fewer than 20% of the embryos derived from *Zar1*^{−/−} females progress to the two-cell stage and show marked reduction in the synthesis of the transcription-requiring complex⁸, and no embryos develop to the four-cell stage. Thus, *Zar1* is the first identified oocyte-specific maternal-effect gene that functions at the oocyte-to-embryo transition and, as such, offers new insights into the initiation of embryonic development and fertility control in mammals.

Pre-implantation embryo development is dependent on stored maternal factors^{1,2}. During meiosis in both sexes, germ-cell genomes are transcriptionally silenced. In the mouse, broad embryonic genome activation occurs at the late two-cell stage^{3,4}. Hence, the oocyte-to-embryo transition depends on maternal transcripts and proteins that accumulate during oogenesis. Despite their many important functions, few mammalian maternal-effect genes have been identified^{9–12}.

To identify novel oocyte-specific genes, we used subtractive hybridization and cDNA library screening to clone a novel gene called zygote arrest 1 (*Zar1*), which shows partial homology to a few egg-expressed expressed-sequence tags (AU023153, BB704019). The full-length *Zar1* cDNA encodes a protein of 361 amino acids (Fig. 1). RT-PCR (Fig. 2a) and northern-blot analyses (data not shown) identified a 1.4-kb *Zar1* transcript

only in the ovary. *In situ* hybridization analysis showed high levels of *Zar1* in growing oocytes of ovaries from wild-type mice (Fig. 3a,b) and from mice lacking the gene encoding growth differentiation factor 9 (*Gdf9*; ref. 13; Fig. 3c–f). Consistent with the expressed-sequence tag findings, we were able to amplify *Zar1* mRNA from wild-type oocytes and one-cell embryos. *Zar1* mRNA was much less abundant in two-cell embryos and was absent in embryos from four-cell through blastocyst stages and in *Zar1*^{−/−} oocytes (Fig. 2b). Thus, *Zar1* mRNA is specifically synthesized in oocytes.

To clone the *Zar1* gene, we screened genomic libraries and characterized the recovered clones. *Zar1* and a related pseudogene (*Zar1-ps1*) contain four exons and map to Chromosome 5. *Zar1-ps1* contains a gap of 13 nucleotides in exon 1, which is predicted to result in a frameshift and protein truncation in exon 2. RT-PCR with *Zar1*-specific primers confirmed that *Zar1* was ovary-specific, but *Zar1-ps1* could not be amplified, establishing it as a pseudogene.

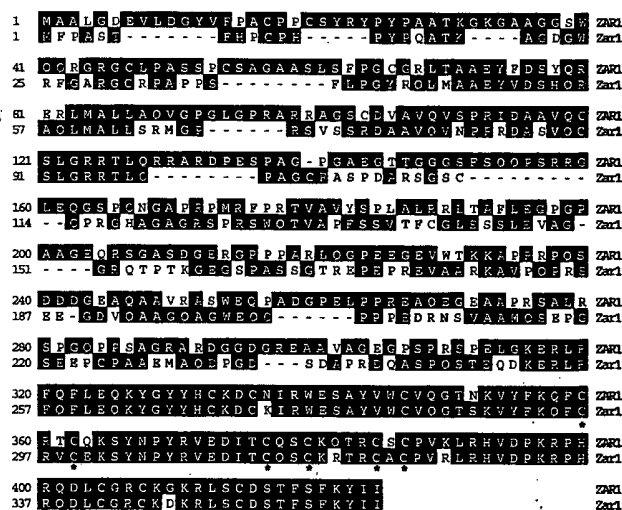
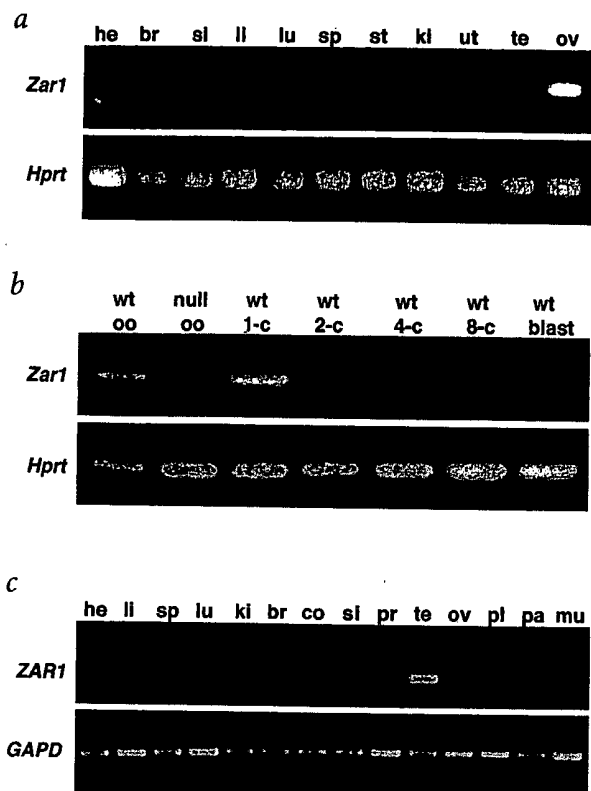


Fig. 1 Comparison of human *ZAR1* and mouse *Zar1* amino-acid sequences. Highest shared identity was observed in the C termini of these proteins. Asterisks denote the cysteine residues of an atypical PHD motif as predicted from ScanSite²⁷.

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To identify the human *ZAR1* ortholog, we carried out BLAST searches and genomic-library screens. *ZAR1*, located on Chromosome 4 in a region of conserved synteny to that on mouse Chromosome 5 containing *Zar1*, spans 4.1 kb and contains four exons, which share 50%, 86%, 84% and 78% nucleotide identity with *Zar1* exons 1–4, respectively. No human pseudogene was found.

ZAR1 is transcribed exclusively in ovary and testis (Fig. 2c). *ZAR1* mRNA is >1.3 kb and encodes a protein of 424 amino acids. *ZAR1* and *Zar1* proteins share 59% amino-acid identity (Fig. 1), and the C termini, encoded by exons 2–4, are highly conserved (91% shared identity). Although the public database lacks *ZAR1* homologs, an atypical PHD motif was discovered in the conserved C termini of *ZAR1* and *Zar1*. PHD sequences are typically characterized by a C_4 -H- C_3 (C -X₂- C -X₍₉₋₂₁₎- C -X₍₂₋₄₎- C -X₍₄₋₅₎-H-X₂- C -X₍₁₂₋₄₆₎- C -X₂- C) zinc-binding amino-acid arrangement⁷. This motif is conserved in the *ZAR1* and *Zar1* C termini but is a C_8 pattern (C -X₂- C -X₁₃- C -X₂- C -X₄- C -X₁- C -X₁₇- C -X₂- C). A C-to-H substitution is also present in the disease-associated PHD-containing protein ATRX¹⁴. Mutagenesis of the H to C in PHD-containing protein KAP-1 has a minimal effect, suggesting that these amino acids are functionally similar¹⁵. PHD domains are found in two major classes of proteins: (i) transcriptional activators, repressors or cofactors and (ii) subunits of complexes that modulate chromatin. Thus, *ZAR1* may be a transcriptional regulator, and the conserved *ZAR1* C terminus is probably functionally important.

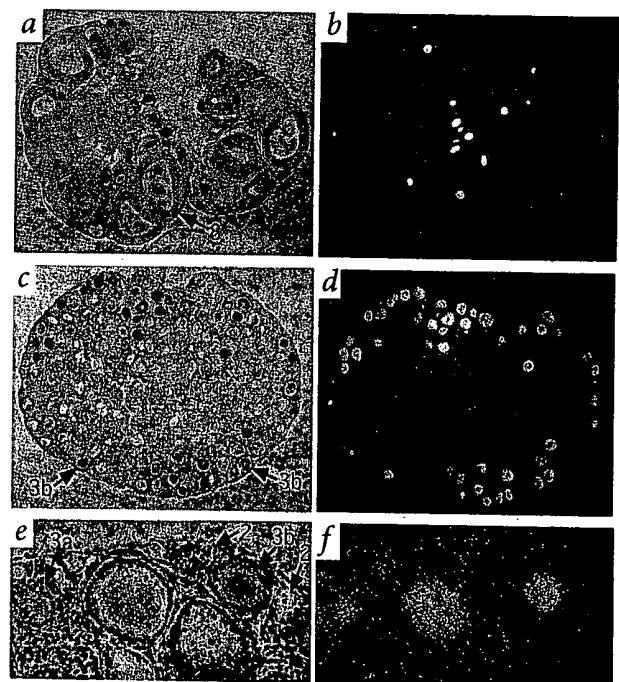
Fig. 3 Localization of *Zar1* in mouse ovaries. Expression of *Zar1* in wild-type (a,b) and *Gdf9*^{-/-} (c-f, ref. 13) ovaries treated with PMSG was analyzed by *in situ* hybridization with a specific antisense probe. Bright-field (a,c,e) and corresponding dark-field (b,d,f) images of each ovary section are presented. Areas of panels c and d are shown at higher magnification in e and f. *Zar1* mRNA was detected at early primary follicle (type 3a) through antral follicle (type 8) stages, but not in primordial follicles (type 2). The follicle classification is based on Pedersen and Peters²⁹.

Fig. 2 Expression analysis of zygote arrest 1 in mouse and human tissues. a, RT-PCR analysis of several mouse tissues showed a *Zar1*-specific 331-bp band only in ovary (ov). b, RT-PCR analysis of wild-type (wt) and *Zar1*^{-/-} (null) oocytes (oo) and wild-type one-cell (1-c) through blastocyst- (blast) stage embryos detected *Zar1* mRNA in wild-type oocytes and one-cell and two-cell embryos. c, RT-PCR analysis of human tissues amplified a fragment of 530 bp. *Hprt* and *GAPD* were used as internal controls for the mouse and human RT-PCR, respectively, as described^{25,28}. The function of *ZAR1* in the testis (te) is unknown. br, brain; lu, lung; he, heart; st, stomach; sp, spleen; li, liver; si, small intestine; ki, kidney; ut, uterus; co, colon; pr, prostate; pl, placenta; pa, pancreas; mu, muscle.

To determine *Zar1* function, we generated *Zar1*-null (*Zar1*^{tm1Zuk}) mutant mice (Fig. 4). Intercrossing F1 heterozygotes (Fig. 4b) yielded 232 F2 progeny (52 wild-type (22.5%), 119 heterozygous (*Zar1*^{+/-}; 51.5%) and 60 homozygous-null (*Zar1*^{-/-}) mice (26.0%)) from 32 litters. Thus, the mutated allele was transmitted with the expected mendelian frequency of 1:2:1. Northern-blot analysis showed a reduction in abundance of *Zar1* mRNA in *Zar1*^{+/-} ovaries and was not able to detect the 1.4-kb *Zar1* mRNA in *Zar1*^{-/-} ovaries (Fig. 4c), confirming that the *Zar1*^{tm1Zuk} allele was null.

Zar1^{+/-} and *Zar1*^{-/-} male and female mice showed no gross or histological abnormalities. To test the fertility of *Zar1*^{+/-} and *Zar1*^{-/-} C57BL/6J129S6/SvEv hybrid mice, we mated them for six months. Consistent with ovary-specific expression of *Zar1*, *Zar1*^{-/-} males were fertile (7.4 ± 0.4 pups per litter). Mating of 14 *Zar1*^{+/-} females with *Zar1*^{+/-} males resulted in 80 litters (0.95 litters per month per mouse; 7.9 ± 0.3 pups per litter), similar to wild-type matings (8.4 ± 0.2 pups per litter; ref. 16). In contrast, breeding of 20 *Zar1*^{-/-} females yielded no offspring over six months. Likewise, ten *Zar1*^{-/-} 129S6/SvEv inbred females bred over six months were also infertile. Thus, *Zar1* is essential for female fertility.

To further define *Zar1* function, we evaluated expression and subcellular localization of *Zar1* in oocytes and early embryos. Consistent with the RT-PCR data, we identified a 45-kDa *Zar1*-specific band only in mouse ovary by western-blot analysis (Fig. 5a). *Zar1* localized predominantly to the cytoplasm of oocytes of wild-type (Fig. 5b,c) and *Gdf9*^{-/-} ovaries (Fig. 5d) and was present from primary through antral follicle stages (Fig. 5e). *Zar1* was distributed diffusely throughout the cytoplasm of fully grown oocytes isolated



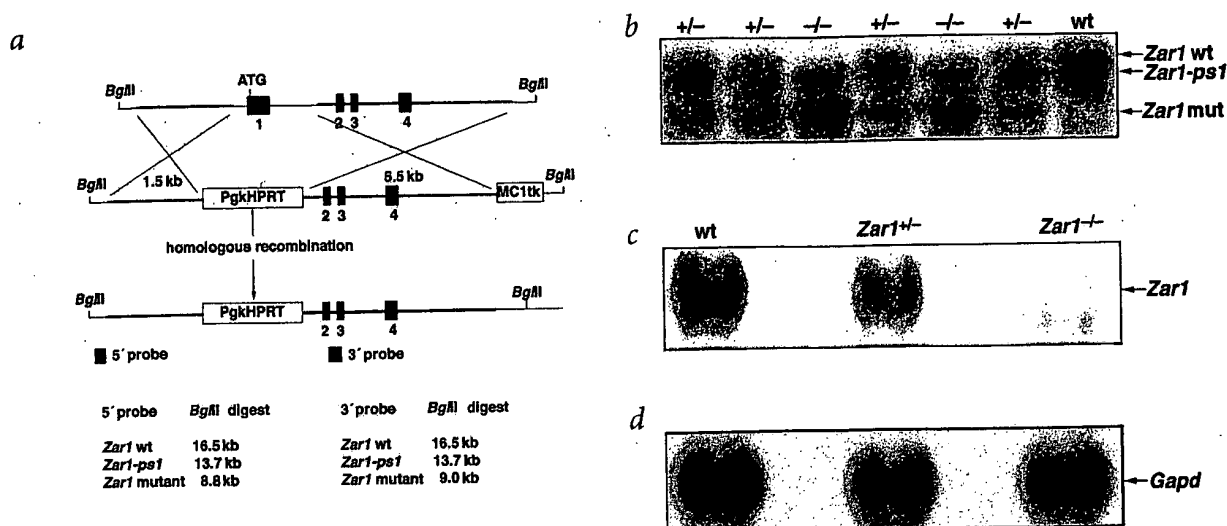


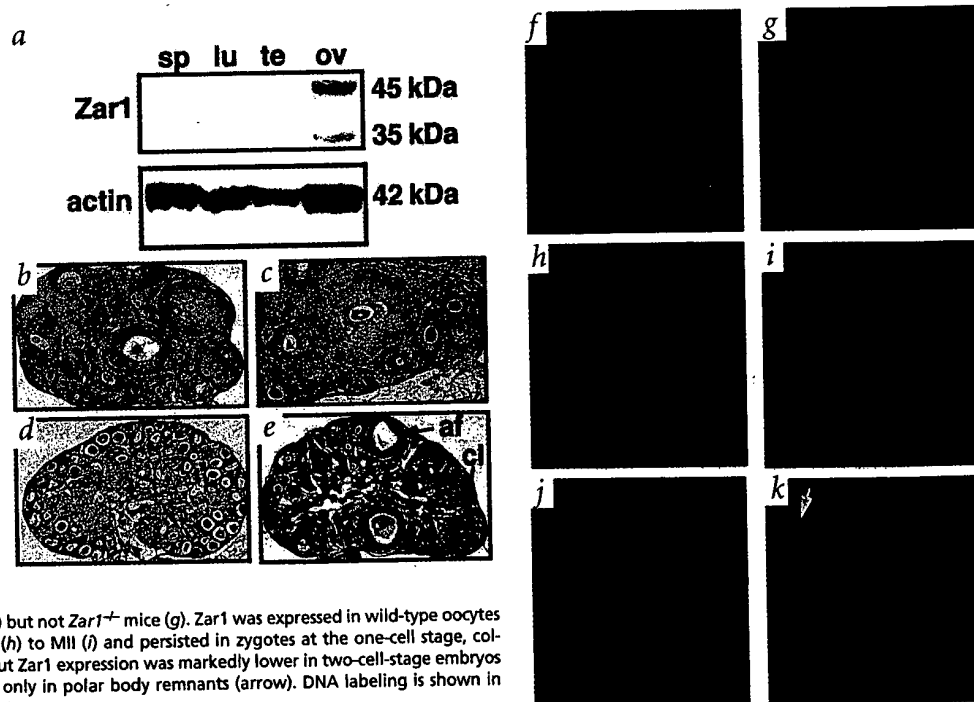
Fig. 4 Targeting strategy of *Zar1* and Southern-blot and northern-blot analyses of F2 offspring. **a**, A targeting vector was constructed by replacing exon 1 (the largest exon, which contains the initiation ATG) and part of intron 1 with a PgkHPRT expression cassette. The MC1tk expression cassette was used for negative selection. Targeted ES cell clones containing a wild-type (wt) allele, a pseudogene allele (*Zar1-ps1*) and a mutated (mut) allele were confirmed by Southern-blot analysis²² and injected into blastocysts to produce chimeric male mice^{22,23}, which were bred to produce F1 *Zar1*^{+/+} offspring. **b**, Southern-blot analysis of genomic DNA derived from seven offspring of one litter from *Zar1*^{+/+} intercrosses. Similar numbers of male and female homozygotes were born. Southern-blot analysis with an exon 1 probe detected only the pseudogene in DNA derived from *Zar1*^{-/-} mice. **c**, Northern-blot analysis of ovarian mRNA from wild-type, *Zar1*^{+/+} and *Zar1*^{-/-} females using the full-length *Zar1* cDNA. On longer exposure, a smaller transcript of unknown relevance was observed in *Zar1*^{+/+} ovaries. The expression level of the 1.4-kb *Zar1* mRNA in wild-type mice was approximately twice the levels present in *Zar1*^{+/+} ovaries. **d**, *Gapd* was used as a control for equivalent loading on the northern blot.

from *Zar1*^{+/+} mice (Fig. 5f) but was not present in ovaries (Fig. 5e) and oocytes (Fig. 5g) from *Zar1*^{-/-} females. *Zar1* was also detected after resumption of meiosis through progression to metaphase II (Fig. 5h,i). *Zar1* persisted in one-cell embryos (Fig. 5j) but was markedly less abundant in two-cell embryos (Fig. 5k). Thus, *Zar1* could function in growing oocytes through formation of two-cell embryos. The rapid disappearance of *Zar1* at the two-cell stage, however, suggests a critical role in the oocyte-to-embryo transition.

To determine the cause of the infertility in female *Zar1*^{-/-} mice, we examined ovarian histology. Ovaries from *Zar1*^{-/-} females

(Fig. 5e) were indistinguishable from those of control females. All stages of follicle development and corpora lutea (indicative of ovulation) were evident in ovaries of *Zar1*^{-/-} females. Moreover, superovulation resulted in similar numbers of oocytes from *Zar1*^{+/+} (31.6 ± 4.7 ; $n = 8$) and *Zar1*^{-/-} (34.3 ± 4.1 ; $n = 14$) females. Most *Zar1*^{+/+} ($74.4 \pm 5.5\%$; $n = 156$) and *Zar1*^{-/-} oocytes ($62.9 \pm 4.3\%$; $n = 137$) resumed meiosis and progressed to metaphase II during a 17-h culture. Like heterozygous controls ($92.9 \pm 2.1\%$), *Zar1*^{-/-} oocytes formed two pronuclei within 8 h after insemination ($82.4 \pm 7.5\%$). Whereas the first cleavage

Fig. 5 *Zar1* expression. A polyclonal antibody against *Zar1* was used for western-blot (a), immunohistochemistry (b-e) and immunofluorescence analyses (f-k) to detect *Zar1* expression. **a**, *Zar1* was detected only in mouse ovary (ov) and was absent in spleen (sp), lung (lu) and testis (te), unlike actin (positive control). A smaller 35-kDa band was also detected in the ovary and may represent a degradation product. Similar to *Zar1* mRNA, *Zar1* protein expression (red staining) began in oocytes of primary follicles and continued through all follicle stages in wild-type ovaries (b,c). *Zar1* was also detected in *Gdf9*^{+/+} ovaries (d) but not in *Zar1*^{-/-} ovaries (e). *Zar1* (shown in green) was detected predominantly in the cytoplasm of fully grown, prophase I-arrested oocytes from *Zar1*^{+/+} (f) but not *Zar1*^{-/-} mice (g). *Zar1* was expressed in wild-type oocytes during the progression from MI (h) to MII (i) and persisted in zygotes at the one-cell stage, collected 6 h after fertilization (j). But *Zar1* expression was markedly lower in two-cell-stage embryos (k), with bright staining evident only in polar body remnants (arrow). DNA labeling is shown in red. af, antral follicle; cl, corpus luteum.



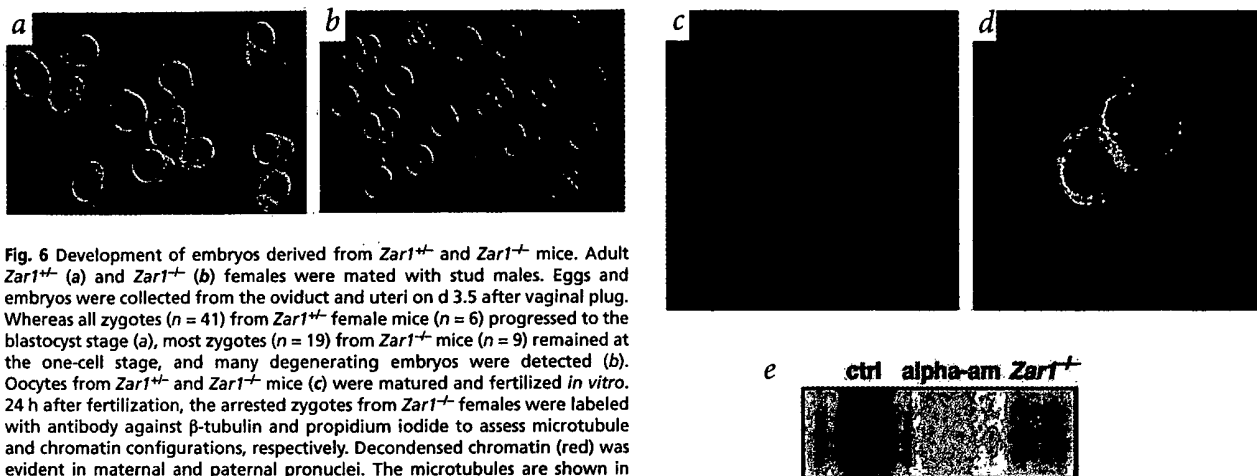


Fig. 6 Development of embryos derived from *Zar1*^{+/+} and *Zar1*^{-/-} mice. Adult *Zar1*^{+/+} (a) and *Zar1*^{-/-} (b) females were mated with stud males. Eggs and embryos were collected from the oviduct and uteri on d 3.5 after vaginal plug. Whereas all zygotes ($n = 41$) from *Zar1*^{+/+} female mice ($n = 6$) progressed to the blastocyst stage (a), most zygotes ($n = 19$) from *Zar1*^{-/-} mice ($n = 9$) remained at the one-cell stage, and many degenerating embryos were detected (b). Oocytes from *Zar1*^{+/+} and *Zar1*^{-/-} mice (c) were matured and fertilized *in vitro*. 24 h after fertilization, the arrested zygotes from *Zar1*^{-/-} females were labeled with antibody against β -tubulin and propidium iodide to assess microtubule and chromatin configurations, respectively. Decondensed chromatin (red) was evident in maternal and paternal pronuclei. The microtubules are shown in green. In a second experiment, the fertilized zygotes were placed in medium with BrdU 8 h after fertilization and cultured overnight (d). Immunofluorescence analysis showed BrdU incorporation in both pronuclei (bright orange) of an arrested zygote from a *Zar1*^{-/-} female, indicative of entry into S phase. e, Early embryos from inseminated *Zar1* null eggs underwent embryonic genome activation. TRC synthesis was detected in two-cell-stage embryos from wild-type control (ctrl) and *Zar1*^{-/-} females. Culture with α -amanitin (α -am, 11 μ g ml⁻¹), an inhibitor of RNA polymerase II, prevented TRC synthesis. The TRC experiments were performed as described⁸ using 15 embryos per lane.

occurred in most *in vivo* (89.3%) and *in vitro* (86.5 \pm 1.4%) fertilized embryos from *Zar1*^{+/+} females, we observed two-cell embryos (some of which appeared fragmented) in only 19.1 \pm 9.1% (*in vitro*) and 20.8 \pm 1.5% (*in vivo*) of embryos from *Zar1*^{-/-} females. 100% of embryos isolated from uteri of *Zar1*^{+/+} females developed to blastocysts by day 3.5, and most cultured control embryos progressed to morula and blastocyst stages (82.1 \pm 1.5%), but we observed only fragmented one-cell embryos and a single two-cell embryo in *Zar1*^{-/-} females (Fig. 6a,b). Therefore, an arrest at the zygote and two-cell stages accounts for the infertility of *Zar1*^{-/-} females.

Additional analysis of the embryonic block showed that *Zar1* null zygotes progressed through G1 and successfully entered S phase. Both pronuclei had decondensed chromatin and incorporation of 5-bromo-2-deoxyuridine (BrdU; Fig. 6c,d). The microtubule network showed an interphase configuration with no assembled spindle apparatus. *In vitro* fertilized oocytes were treated with colcemid, which depolymerized microtubules and arrested all zygotes from *Zar1*^{+/+} females at M phase. Only a few *Zar1* null zygotes were similarly arrested, but this number corresponded to the small percentage of *Zar1* null embryos that entered M phase and became two-cell embryos, indicating that most arrested earlier at the S or G2 stages. Thus, the maternal and paternal genomes remained separate in discrete pronuclei, and the two haploid genomes did not unite, indicating that the fertilization had not been completed.

To further define the block in the *Zar1* null embryos, we analyzed the synthesis of a group of proteins called the transcription-requiring complex (TRC), markers of embryonic genomic activation⁸. Although TRC proteins were detected in the limited number of *Zar1* null embryos that reached the two-cell stage, their TRC expression levels were 15% of those of the control embryos, suggesting that there are defects in embryonic genome activation (Fig. 6e). The precise mechanisms by which *Zar1* directs the oocyte-to-embryo transition have yet to be determined.

The decrease in *Zar1* expression in normal two-cell embryos correlates with the usual rapid degradation of maternal transcripts after fertilization¹⁷. Developmental processes ongoing at this time include completion of the meiotic-to-mitotic cell-cycle transition and activation of the embryonic genome¹⁻⁴.

Notably, inhibition of embryonic transcription does not prevent the first mitotic division but arrests embryos beyond the two-cell stage¹⁷. Of the other maternal-effect genes⁹⁻¹², only *Mater* (maternal antigen that embryos require) is expressed exclusively in oocytes and early pre-implantation embryos. Compared with *Zar1* null embryos, *Mater* null embryos block at the two-cell stage and have higher levels of genome activation. The predominant one-cell-stage arrest of fertilized eggs from *Zar1*^{-/-} females suggests that this maternal factor functions before embryonic genome activation. Although both *Mater* (53%) and *Zar1* (59%) share low identity with their human orthologs¹⁸, these human orthologs probably have conserved functions in early embryogenesis and fertility. Identification of proteins that interact with *Zar1* may provide insights into the roles of this maternal protein in regulating the essential transition from oocyte to embryo. This study presents the first identification and characterization of zygote arrest 1, a gene expressed during oocyte development, the function of which is key for the initiation of embryogenesis.

Methods

mRNA expression analysis. We obtained total RNA from mouse tissues or embryos using the RNA STAT-60 method (Leedo Medical Laboratories). We carried out *in situ* hybridization with a *Zar1*-specific probe as described^{19,20}. We carried out RT-PCR analysis of mouse and human cDNAs (primer sequences are available upon request).

Mouse ovary cDNA library and genomic library screening. We used a *Zar1* cDNA probe (corresponding to nucleotides 896–1,220 of the full-length *Zar1* cDNA) to screen a wild-type mouse ovary cDNA library. We used the full length 1,259-nt *Zar1* cDNA, which contains an open reading frame from nt 28–1,110, to screen 129S6/SvEv mouse genomic λ FixII phage and human genomic DNA libraries (Stratagene). We also used *Zar1*-specific primers to screen a 129X1/SvJ BAC library (Genome Systems). We hybridized filters as described²¹.

ES-cell manipulation and Southern-blot analysis. We electroporated the linearized *Zar1* targeting vector into the HPRT-negative AB2.2 ES-cell line, selected clones in hypoxanthine, aminopterin and thymidine and 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-iodouracil and screened DNA from the clones by Southern-blot analysis. We found that 5 of 171 (3%) of the ES cell clones analyzed were targeted at the *Zar1* locus. ES cell line *Zar1*-140-G11 was injected into blastocysts to produce chimeric male mice

that were fertile and transmitted the mutated *Zar1* allele (*Zar1^{tm1Zuk}*, here-in called *Zar1⁻*) to F1 progeny as described^{22,23}.

Superovulation, mating and *in vitro* embryo culture. We injected 25-d-old *Zar1^{+/+}* and *Zar1^{-/-}* female mice with pregnant mare serum gonadotropin (PMSG; intraperitoneal injection of 5 IU per mouse) and gave them human chorionic gonadotropin (intraperitoneal injection of 5 IU per mouse) 48 h later. Mice were then caged overnight with (C57BL/6) × 129S6/SvEv F1 stud males. The following morning, we recovered eggs and embryos in M2 medium, counted them and cultured them *in vitro* up to 4 d in M16 medium (Sigma). Alternatively, adult mutant females were mated to stud males, uteri and oviducts were flushed on d 1.5, 2.5 or 3.5, and embryos were collected and cultured in M16 medium.

***In vitro* oocyte maturation and fertilization.** We injected sexually mature *Zar1^{+/+}* and *Zar1^{-/-}* female mice with 5 IU of PMSG to stimulate pre-ovulatory follicle development. We isolated cumulus-enclosed oocyte complexes 48 h later and cultured them for 17 h in minimal essential medium with 5% serum. We subsequently removed the surrounding somatic cells and examined the oocytes to determine the progression of meiosis. Mature MII-stage eggs were inseminated *in vitro* with sperm from wild-type (C57BL/6J × SJL/J) F1 mice²⁴. We assessed development of zygotes and two-cell-stage embryos 6 and 24 h after fertilization, respectively. We evaluated blastocyst formation on d 5.

Production of polyclonal antibodies against *Zar1* and immunostaining. We subcloned a partial mouse *Zar1* cDNA (nt 178–1,083 corresponding to amino acids 51–352) into pET23b vector (Novagen) and injected chimeric recombinant *Zar1* protein (with a T7 tag at the N terminus and His tag at the C terminus) into goats to produce polyclonal antibodies (CoCalico Biologicals). We carried out western-blot analysis and immunostaining as described²⁵ using primary antibody (diluted at 1:1,000). For western-blot analysis, we used secondary antibody against goat conjugated with horseradish peroxidase (Boehringer Mannheim; diluted at 1:2,000) and ECL Western Blotting Detection Reagents (Amersham Biosciences). For immunostaining, we carried out incubation with secondary antibody and visualization of positive cells using the New Fuchsin kit (BioGenex). We used pre-immune serum in control sections.

Immunofluorescence. We carried out immunofluorescence analysis of oocytes and embryos as described²⁵. We carried out reactions with the goat *Zar1* antisera (diluted at 1:1,000 in block solution) for 1 h and then exposed samples to 3 µg ml⁻¹ of antibody against rabbit IgG conjugated with fluorescein isothiocyanate (Jackson Immuno Research Laboratories) for 45 min. We labeled DNA with propidium iodide (10 µg ml⁻¹ for 10 min). We evaluated negative controls with pre-immune serum.

We evaluated fertilized zygotes that had not undergone the first mitotic division by 24 h after fertilization to determine chromatin and microtubule configurations. The zygotes were fixed, permeabilized and blocked. We carried out all subsequent steps, including rinses, at 37 °C in block solution. We labeled microtubules with antibody against β -tubulin (3.8 µg ml⁻¹ for 1 h) and secondary antibody against mouse conjugated with fluorescein isothiocyanate (1.3 µg ml⁻¹ for 45 min) and labeled DNA with propidium iodide as above. We detected fluorescence using a confocal microscope.

DNA synthesis. Fully-grown oocytes from *Zar1^{+/+}* and *Zar1^{-/-}* mice were *in vitro* matured and fertilized as above. Approximately 8 h after fertilization, we transferred the zygotes that formed male and female pronuclei to medium supplemented with 50 µM BrdU for overnight culture. 24 h after fertilization, embryos were fixed and processed to assess BrdU incorporation²⁶. We detected fluorescence using a confocal microscope.

Statistical analysis. We calculated statistical significance by one-way ANOVA. Data are expressed as mean ± s.e.m.

URL. ScanSite²⁷ is available at <http://scansite.mit.edu>.

GenBank accession numbers. *Zar1* cDNA, AY191415; *Zar1* gene, AY193889; *ZAR1* cDNA, AY191416; *ZAR1* gene, AY193890.

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Competing interests statement

The authors declare competing financial interests: see the Nature Genetics website (<http://www.nature.com/naturegenetics>) for details.

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